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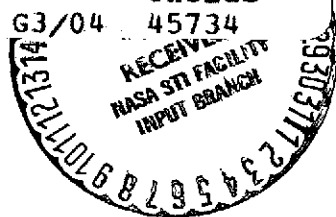
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16. Abstract   The nature of the phenomena responsible for the need for activation energy in a chemical reaction is examined. These phenomena basically result from electrostatic interactions, and there is no way to decrease the total work required to overcome these interactions, but work can be supplied in several steps. The fractioning of a reaction induced by the intervention of an enzyme substantially accelerates its rate due to the exponential decrease in the number of molecules with their energy level of thermal agitation. An example is given for arbitrary free activation energy values. The chief function of an enzyme is to ensure satisfactory coordination of successive steps.

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THE ENERGY ASPECT OF THE MECHANISM OF ENZYME ACTION

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In order to explain the efficiency with which enzymes /21* catalyze reactions whose rate in the absence of these enzymes would be zero, at least according to our methods of investigation, frequently a systematic attempt is made to determine the mysterious property which enables them to "lower the activation energy barrier." This approach to the problem has a basic flaw in that it implicitly postulates a given type of mechanism and masks the significance of an essential characteristic of catalytic reactions, namely that such a reaction always occurs in several steps. Now, it is easy to show that merely breaking a reaction down into several steps is sufficient to accelerate its rate considerably for a single level of activation energy furnished to the total reaction, but the importance of this finding, which is fairly obvious [1], is not generally recognized. The present discussion is intended to show that it is impossible to consider that an enzyme decreases the total quantity of activation energy to be furnished in sufficient proportions to explain its high catalytic capability, and that the primary function of an enzyme is to coordinate a series of reactions.

Principal Factor Limiting the Rate of a Reaction

It is necessary to begin by reviewing the reason for which the rate of an exergonic reaction is not limited solely by the total number of encounters of molecules capable of reacting. An explanation for the ineffectiveness of most encounters was found by Arrhenius [2] on the basis of his experiments on variations in

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* Numbers in the margin indicate pagination in the foreign text.

the rates of reactions with temperature. The theory based on this explanation was subsequently constructed by Eyring [3], who, developing an idea proposed by Marcelin [4], extended it to both directions of a reaction and termed it the "theory of absolute rates." This theory led to a change in the expression for the rate constant of the reaction given by Arrhenius, without altering the basic initial concept.

It is acknowledged that for an encounter between molecules to be effective, these molecules must possess a given quantity of energy which may be manifested by a high value of interaction energy between an electron and an atom, vibration energy for two atoms, translation or rotation energy, or, most frequently, energy in several of these different forms at once. In general, the molecules may be assumed to be distributed among the different energy levels accessible to them according to the Maxwell-Boltzmann law: almost all of them are at the lowest level corresponding to the temperature of the whole, termed the "fundamental level." Without exception, two molecules must be at a higher level in order to react with each other. In studying the rate of a reaction one does not observe the individual behavior of the molecules, and only statistical averages may be taken into consideration. The absorption or release of energy resulting from changes in the distribution of atoms among the various levels and forms of energy must be taken into account, when these changes result from variations in the number of complexings available to the atoms, or, in other words, variations in their entropy. The number of complexings available is especially important, with regard to the probability of favorable orientation of each of the reactant particles in relation to each other and the angle of their trajectories at the moment of encounter. As a result, one is led to the conclusion that in order for molecules to react, they must be brought to a level of free energy higher than the fundamental level; or for biochemical reactions, to a higher level of free

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enthalpy, since these reactions almost always occur at atmospheric pressure, which is virtually constant. Molecules which at least possess the additional energy necessary in order to react are termed "activated." The difference between the fundamental level and the level of the activated molecules, normalized to 1 mole at unit activity in the fundamental state and in the active state, is termed the free enthalpy of activation, ΔG^{+}. It is stated that molecules capable of reacting with each other must cross this "energy barrier" in order to yield the products of the reaction.
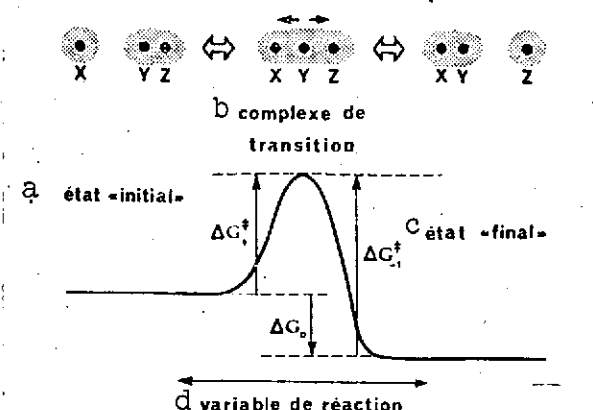

Fig. 1. Schematic representation of the passage of reactant molecules to the transition complex state by acquisition of free enthalpy of activation. The upper part of the figure represents a diatomic molecule exchanging one of its atoms for a free atom, since this example is examined later on. However, the graph representing the variations in energy level of the reactant substances as a whole is valid for all cases.

Key: a. "Initial" state; b. Transition complex; c. "Final" state; d. Variable of reaction

The free enthalpy of activation for some reactions may be supplied to the molecules by photons. These reactions thus fall within the category of photochemical reactions. However, it must be noted that, in the direction in which the reaction is exergonic, the electromagnetic wave furnishes only the free energy necessary for the molecules to cross a "barrier" and that this energy will be returned to the environment at the end of the reaction.

In most cases, the activation energy is acquired by a molecule fortuitously during collisions with other molecules provoked by Brownian agitation. The aleatory

exchanges of energy between molecules generate continual fluctuations in the distribution of thermal agitation energy among the molecules. At the ambient temperature, these fluctuations are not adequate to change the energy level of an electron (the corresponding energy quantum, which is on the order of 30 kcal, is too high), with the result that the energy fluctuations of the molecules involve only their translational and angular velocities and the amplitude of vibration of their atoms. On an initial approximation, the proportion of molecules located at a level of thermal agitation energy at least equal to $\Delta G^{\ddagger}$, per mole, above the fundamental level is $e^{-\Delta G^{\ddagger}/RT}$, where R is the ideal gas constant and T the absolute temperature. The theory of absolute rates informs us that the rate constants k_1 and k_{-1} in either direction of a bimolecular reaction are given by the equations:

$$k_1 = \varkappa \, \frac{2\,k\,T}{h} \, e^{-\frac{\Delta G^{\ddagger}_1}{RT}}$$

and

$$k_{-1} = (1-\varkappa)\frac{2\,k\,T}{h}\, e^{-\frac{\Delta G^{\ddagger}_{-1}}{RT}}$$

where k is the Boltzmann constant, h the Planck's constant and $\varkappa$ a factor termed the transmission factor. $\varkappa$ is the proportion of transition complexes which fall below the "activation" energy level in the final state; $\varkappa$ may thus be assumed to be between 0 and 1. Since the chances are equal that any given transition complex will reproduce the original substances as that it will yield the final products, $\varkappa$ here will be equal to 0.5 (rather than 1, which is the appropriate value when the factor 2 is not disclosed).

Free activation energies are always counted positively, no matter what the direction of the reaction may be, since they always correspond to a change from a fundamental state, whether it is "initial" or "final," to an activated state.

$\Delta G_1^{+\ddagger}$ and $\Delta G_{-1}^{+\ddagger}$ are normalized to the unit activity of the constituents; as a result their difference is equal to the standard free enthalpy variations of the reaction, ΔG_o (Fig. 1).

It may be seen that the rate constant in one direction is independent of the activation energy of the reaction in the opposite direction. This is the reason for which the theory was termed the "theory of absolute values."

The following table gives these values in round numbers for the factor $e^{-\Delta G^{\ddagger}/RT}$ at 20°C for a series of values $\Delta G^{+\ddagger}$ expressed in kilocalories.

$\Delta G^{\ddagger}$	1	5	10	15	20	25	30	35
$e^{-\frac{\Delta G^{\ddagger}}{RT}}$	$1,8 \times 10^{-1}$	2×10^{-4}	$4,1 \times 10^{-8}$	$8,3 \times 10^{-12}$	$1,7 \times 10^{-15}$	$3,43 \times 10^{-19}$	7×10^{-23}	$1,4 \times 10^{-25}$

It may be seen that this factor decreases extremely quickly as $\Delta G^{+\ddagger}$ increases. The rate of a reaction diminishes very rapidly as the free enthalpy of activation increases and becomes negligible for approximately 30 kcal, despite the high value of the factor kT/h (nearly $6 \cdot 10^{12}$ sec^{-1} at 293°K).

The value of the free enthalpy of activation in one direction, from left to right, for example, may be derived directly from the equations given above by replacing k_1 with the rate constant k determined experimentally under conditions in which the rate of the reverse reaction is negligible.

Most reactions which occur in living things would be much too slow to support life at the temperatures prevalent on the earth if they were not considerably accelerated by extraordinarily efficient catalyzers, the enzymes. Attention should be drawn to the fact that, as a catalyst, an enzyme remains intact at the end

of the reaction; consequently it cannot have furnished any energy to the reaction in a definitive sense.

Nature of Phenomena Responsible for the Need for Activation Energy

Before attempting to explain the mechanism of enzyme action, it is first necessary to know why molecules must be supplied with energy in order to react. Only when we know the exact use of this energy can we hope to understand the role played by an enzyme. This question is relatively easy to answer in the case of a diatomic molecule exchanging one of its atoms for a free atom (Fig. 1) according to the diagram:

$$X + YZ \overset{\rightarrow}{\leftarrow} XY + Z.$$
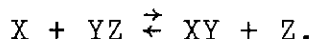
Eyring considered the simple case in which the three atoms are hydrogen atoms in a gaseous state. This example subsequently appeared to raise some problems since the transmission factor involved must be extremely small. Here only the qualitative aspect of Eyring's reasoning will be retained, since this aspect was not called into question. There will be no attempt to stipulate the nature of the atoms, assuming only that molecules XY and YZ are stable, but molecule XYZ is not, and, in order to simplify the discussion, that the charge of Y is of opposite sign to those of X and Z, with the result that no activation energy need be supplied to form bonds between X and Y or Y and Z.

When the vibration energy of the bond in the molecule YZ has a value which statistically corresponds to its fundamental thermal agitation level at the temperature of the medium, assumed here to be the level of origin, the amplitude a_o of the relative vibrations of the two atoms is slight (Fig. 2). The two atoms may be separated from each other only if a considerable force is exerted on them, while they will separate spontaneously if they are

brought to the level w_r. Additionally, at this temperature, the translation energy of most of the molecules and atoms is too low for the electron clouds to interpenetrate, during a collision be- tween a molecule and an atom, to the point where a bond is trans- ferred from one atom to another. Cleavage of the existing bond and the establishment of a new bond thus necessitates that all three of the reactant atoms reach a higher energy level than the fundamental level, which may occur as the result of aleatory collisions. It may be demonstrated that the work required for the removal of the bonded atoms and the approach of the isolated atom is minimum when the three atoms are aligned (Fig. 1). An accidental separation of the YZ nuclei as a result of the chance acquisition of vibration energy greater than the average will facilitate an initial approach of X and Y as the result of the chance acquisition of a relative translational velocity which is properly oriented, reciprocal, and higher than the average. If the energy level of all three atoms is adequate, they may reach the "activated complex" or "transition complex" state. In this state, the interactions between the nuclei and electrons of atoms X and Y are exactly equivalent to the interactions between the nuclei and electrons of atoms Y and Z. The three atoms do not form a molecule because this state is unstable, and the system either returns to its previous state or enters a new state. Here for a unit activity an amount of energy is released which, in the first case, is exactly equal to that which had to be acquired by atom X and molecule YZ to form the activated complex from the fundamental state, that is $\Delta G_1^{\ddagger}$, and in the second case, is equal to $\Delta G_1^{\ddagger} + \Delta G_0^{\ddagger} = \Delta G_{-1}^{\ddagger}$, the free energy necessary to bring the "products" of the reaction, XY and Z, to the transition complex state. This simple example clearly demonstrates the necessity for a temporary acquisition of energy and the use made of this energy to overstrain the bond between Y and Z and facilitate the interpenetration of the electron clouds of X and Y.
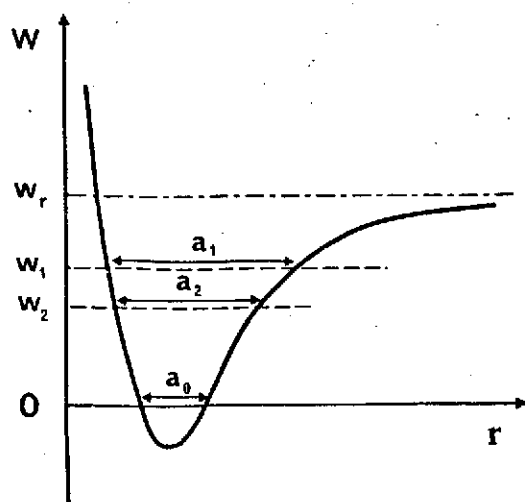
Fig. 2. Morse curve representing the mutual energy of two bonded atoms. The abscissa represents the distance between the two nuclei, and the ordinate represents their energy level adjusted to an arbitrary level (here, the lowest level at the temperature of the medium). The a factors are the amplitudes of vibration of the two atoms in relation to each other at the corresponding energy level.

One may also consider the case of this process occurring in two steps: the decomposition of molecule YZ into two individual atoms during an initial stage is subsequently followed by the combination of Y with X. In this case, the activation energy will be the energy necessary to raise the vibration energy to the level w_r; this energy may be obtained only by the thermal agitation of atoms YZ alone. Once atom Z has been released, atom Y is able to form a bond with atom X as soon as it encounters this atom. In this case, the nature of the reaction requires molecule YZ to use fluctuations in its thermal energy to cross a higher energy barrier than that which was sufficient when atom X was also contributing energy.

A comparison of the two processes clearly shows that the activation energy in the first process is equal to the energy necessary to overcome the attraction between the two atoms, which have lost the energy released by a concomitant related event: here, the translation energy resulting from a fortuitously high relative velocity for the two reacting entitites.

Other cases should be considered, including those in which the energy may be furnished by the attraction of opposite signs

for the two atoms approaching each other and, on the other hand, those in which an energy of "activation" may be necessary to permit an interpenetration of electron clouds, which would have no chance of occurring spontaneously in the fundamental state.

The situation is usually extremely complex and is virtually impossible to analyze, since the two atoms exchanging a third a re bonded to other atoms and each undergo a number of interactions.

In any case, the forces exerted between two atoms are always of electrostatic origin, even when they are expressed mathematically by "exchange energy" terms.

In order for a reaction to occur, it will thus be necessary in all cases that the electrostatic interactions existing within molecules in a fundamental state be overcome. The activation energy will be the energy necessary to obtain this result, with subtraction of the energy which may simultaneously arise from other interactions and the thermal agitation of the molecules brought into play in the fundamental state. When the energy required is greater than the energy which may be furnished on the average by simultaneous phenomena, most of the molecules will remain in the initial state. Reactions will occur only among those few molecules which undergo a fluctuation in their thermal energy which increases the vibration energy of the bonds involved and the translation energy of the molecules to the point that they enter a state of stable equilibrium between bonds being broken and being being formed: the transition state.

It is not known if there is an equal probability that the transition complex will move toward a new atomic state or, on the other hand, will return to the initial state. This is why the transmission factor κ is introduced into the expression for rate constants.

In the case of atoms of hydrogen gas, it has been computed
that if these atoms are at the energy level corresponding to the
formation of the transition complex, the probability that two
atoms encountering each other will form a hydrogen molecule is
extraordinarily small, since the life of the transition state is
less than that of an interatomic vibration. Under these condi-
tions, the transition coefficient is extremely small. This
phenomenon may easily be understood if one observes that at the
temperature of dissociation of the hydrogen molecule, which of
course is greater than the "temperature" of the transition complex,
in practice two atoms encountering each other do not recombine,
since the molecule formed immediately dissociates. However, as
Glasstone and colleagues have pointed out [5], when a reaction
occurs, on the average, at ordinary temperatures, which are much
lower than the bond dissociation temperature studied in biochemistry,
and when it additionally occurs in solution, the relative shifts of
the mean positions of the atoms are very slow to occur in com-
parison to the vibrational movements of those atoms forming bonds.
Under these conditions, the Eyring theory may be applied without
restriction, and all the transmission coefficients may be assumed
to have a value of 1/2.

In the general case, the problems involved in determining the
nature of the work required by a momentary demand for energy,
which subsequently will be restored in an exergonic reaction, are
much more complex than in the case of three atoms examined above.
We will consider the relatively simple case of a hydrolysis
reaction, that of the peptide bond. The advantage of considering
hydrolysis is that the expressions for the kinetic constants are
simplified, since it may be assumed that the activity of the
water remains constant and equal to unity. This makes it possible
to omit this magnitude from the formulas, making them less
cumbersome.

Generally, only the stiochiometric equation expressing the hydrolytic balance is written:

$$\ldots -\underset{\underset{O}{\overset{\|}{}}}{\overset{\overset{H}{\overset{|}{}}}{C}}-N- \ldots + H_2O \rightleftharpoons \ldots -COOH + NH_2- \ldots \quad (1)$$
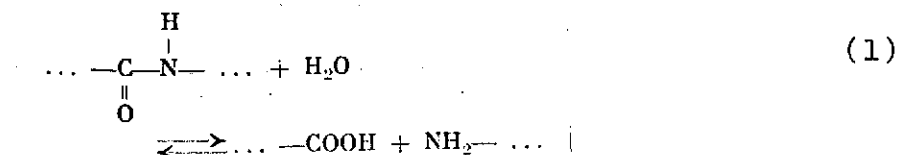
If the reaction actually followed this simple process, due to the short life of the excited states of the molecules, it would be necessary that the weakening of the bonds occur appreciably at the same time as the interpenetration of electron clouds. In other words, the activation energy necessary for several operations belonging to the types considered above must be furnished by Brownian agitation virtually at the same instant. The series of events may thus be represented by the diagram given in Fig. 3. There must simultaneously be weakening of the ... C-N ... and H-OH bonds accompanying the gradual separation of the C and N atoms and the H and O atoms, and the approach of C and O atoms as well as N and H atoms, despite the repulsion of the electron clouds, until the point when a "transition complex" is formed. The chances will be equal (for $\kappa \sim 0.5$) that this complex will yield peptide and the water molecule once again as that it will yield the products of hydrolysis. In this simplified diagram, there is only one way to pass from the initial state to the final state, and thus only one transition state; in order for the molecules to attain this state they must acquire a given level of activation energy all at once.

The electrostatic work necessary to separate or bring together two charges depends only on their initial and final distances from each other; it is independent of the path used. The intervention of an enzyme cannot decrease the work of this type to be furnished in order for the molecule to shift from the
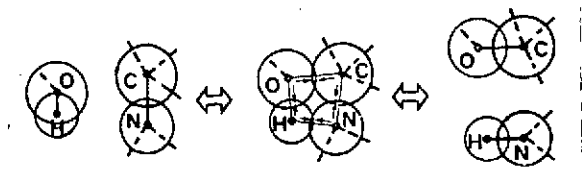
Fig. 3. Schematic representation of the formation of a transition complex during hydrolysis of a peptide bond. Only atoms directly involved in the reconstitution of the bonds are shown. For the sake of clarity, the atoms are represented by circles whose radius is 1/2 the van der Waals radii. The intact water molecule and peptide chain are on the left; the right side of the figure shows the ends of the two fragments formed; and the center shows the transition complex in which the interactions between the four atoms involved reach equilibrium. The latter situation is unstable.

fundamental state to the transition complex state at the outset, in accordance with the process represented by the diagram in Fig. 3.

Possible Roles of an Enzyme

It is possible to weaken electrostatic attraction or repulsion by a suitable arrangement of electrical charges in the vicinity of the atoms involved. Less energy is therefore necessary to overcome /26 these interactions, but the weakening of these interactions will have required an amount of work exactly equal to the work saved. The only difference will be that the work will have been furnished in two steps. It may be assumed that one of the roles of the enzyme is to bring about a new distribution of charges which facilitates reconstitution of the molecules necessary for hydrolysis. First, however, accomplishing this new distribution will require at least the amount of energy which subsequently will be saved, and second, as soon as a new molecule is involved in the reaction the diagram given in Fig. 3 no longer applies.

The first observation which may be made is that the number of reacting particles involved in the reaction is increased by one. It was noted above with reference to the substitution of one atom for another in a hydrogen molecule, that the molecule may dissociate more easily as a result of an encounter with an individual

atom. This is because for a given average level of thermal agitation, the fact that the energy may be furnished by two different reacting entities entrains the necessity for each of them (actually, only the molecule in the example selected) to be raised to an energy level as high as if the molecule were unbonded; that is, the level of free enthalpy of each type of molecule, normalized to 1 mole, may be much lower. When a reaction is catalyzed by an enzyme, one must take into consideration the aleatory excess energy which may be contributed by this additional type of molecule. This contribution is not negligible, since the enzyme molecule has as great a chance of reaching a high energy level as each of the substrate molecules. However, the additional thermal agitation energy due to the intervention of the enzyme can have an influence only during fixation of the initial substrate on the enzyme. Now, in cases where it could be determined, this energy of activation was found to be relatively small in relation to that required by other steps. As a result, although this effect cannot be overlooked, it does not appear to be a determining factor.

The possibility of a momentary concentration of the thermal agitation energy of the enzyme on the bond to be broken is sometimes cited as an explanation for the high catalytic capability of enzymes. In general, when a molecule acquires an energy supplement as the result of a collision, statistically this energy supplement is distributed equally among the vibration energy of its bonds, its energy of translation and its energy of rotation. (At ordinary temperatures, this energy supplement is always too slight to change the energy of the electrons.) However, at any given instant the energy is not equally distributed among the various degrees of freedom and, for the energy of vibration, among the various bonds. A high level of momentary translation energy will facilitate the approach of molecules with charges of the same sign; the acquisition of high energy of vibration by a

bond may facilitate its cleavage when it is already subjected to the influence of another molecule. One can see the possibility that, in a large molecule such as a protein, the breadth of fluctuations in the distribution of energy among the different parts of a single molecule might become considerable. In addition, a specific structure (which furthermore is not identified) would facilitate the confinement of a large part of the agitation energy of an entire enzyme within a specific bond in the substrate which it has fixed. If this supposition were confirmed, it would help explain why enzymes are always large molecules; however, no precise observations have been furnished to support this theory. Furthermore, no data are available on the breadth of the fluctuations of Brownian energy in proteins. This theory poses intriguing problems, but at our current state of advancement it remains too questionable to be cited as an important factor in the effectiveness of enzymes.

Another reason presented for the catalytic power of enzymes is the effect of reciprocal orientation which they impose on the two substrates which are to react with each other [6]. This effect undoubtedly exists, but taking it into account only begs the question, since it presupposes a solution for the problem of the orientation of the substrates in relation to the enzyme prior to their fixation.

E. Pollard [7] has examined the possibilities for favorable reciprocal orientation of two random molecules, each possessing a reactant site. Despite the simplifications which he found necessary, the fact emerges from his analysis that there is a good chance that the molecules will orient themselves favorably in relation to each other as a result of Brownian movement of rotation during the time they remain within the range of van der Waals attractions, which they exert on each other, despite the Brownian movement of translation. In addition, the circumstances

must be more favorable than is indicated by the numerical values pointed out by this investigator, since the macroscopic diffusion constant, which he was forced to take into consideration since it is the only measurable quantity, is dependent on the mean displacement of the molecules over a large distance and does not take into account the "cage effect," which tends to keep two molecules in contact in a fluid as long as one of them does not leave an opening by scattering the molecules in the medium in order to move away from the first molecule.

When a reaction between two types of molecules in the presence of a catalyst is compared with that which would occur in the absence of a catalyst, the first point which should be noted, even though it is obvious, is that two completely different reactions are actually involved. Earlier we have had occasion to observe that the enzyme becomes bonded to one of the substrates, which implies the fact that an enzymatic reaction occurs in several steps, while we initially attempted to treat our example of a hydrolytic reaction as a reaction with only a single phase: passage of the molecules involved from a fundamental state into a single transition state from which they either return to the initial state or proceed to a final state.

In the hydrolytic cases which it was possible to analyze, the process was always found to be relatively complex. Either the water is already dissociated and the C and N atoms of the peptide bond are in the presence of H^+ ions, or rather, H_3^+O, or a high concentration of OH^- ions; or the hydrogen atom is surrendered to the nitrogen by a molecule serving as intermediary (Fig. 4). The dissociation of the water molecule, which necessarily disappears, is not directly linked to the cleavage of the peptide bond and does not necessarily occur at the same time as this cleavage. Since equation (1) gives a correct account of the balance of the reaction, the same bonds definitely must be broken or
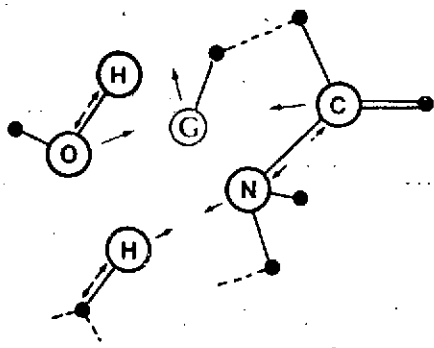
Fig. 4. Schematic representation of relative displacement of atoms actually involved during the hydrolysis of a peptide bond. The left-hand side of the figure shows the water molecule which will supply the oxygen (ionized to neutral pH) and the molecule (whose nature is not precisely determined) which will supply the hydrogen. The right-hand side of the figure shows the peptide chain with individual representation of the N and C atoms whose bond will be broken and an atom G belonging to a lateral chain. This atom, initially located between two atoms (here, O and C), obstructing their movement toward each other by its physical presence alone, must be removed mechanically.

or formed once and for all at a given moment. However, there is nothing to indicate that all these operations must occur simultaneously, as our diagram would imply, nor that they would occur under the same conditions, that is, without passing through one or several intermediate steps.

For purposes of simplification, it will be assumed that the ionization of carboxyl and amide groups, which, under physiological conditions, that is, at neutral pH, occur in the forms COO^- and NH_3^+, occurs in a final step after cleavage of the former covalent bonds and the formation of new bonds. These ionization processes will not be taken into consideration here.

Analysis of the mechanisms of chemical reactions has shown that the existence of intermediate steps between the initial and final steps can seldom be detected, and thus that many reactions should be considered to include several successive steps.

In the case of an enzymatic reaction, we are obliged to assume in all cases that the catalyzed reaction includes at least one more step than the same reaction without catalysis: fixation

of an initial substrate on the enzyme; and probably two steps which are more or less independent of the fixation of the two substrates. The result of this fixation is a modification of the conditions under which the rest of the reaction occurs. Apparently there are only two modes of intervention for an enzyme:

a) During fixation of the enzyme on the substrate, charges are drawn to the immediate vicinity of the atoms involved in the reactions and placed in such a way that they weaken certain bonds or decrease the repulsion between atoms, facilitating subsequent interpretation of their electron clouds. Reconstitution of the electron clouds may additionally result from the direct fixation, by a weak bond and without cleavage of an existing bond, of a new atom on one of the atoms which subsequently will be bonded to an atom in the other substrate.

The work which must be performed to bring these charges into /28 the vicinity of the atoms will be exactly equal to that which will be saved subsequently, but the energy will have been supplied in two steps. At each step the amount of energy which the molecules must acquire will be smaller, and due to the exponential decrease in the number of molecules as a function of their energy level, the proportion of molecules capable of passing through each of the steps will increase considerably.

b) Cleavage of the bond existing in the initial molecule between one of its atoms, A, involved in the reaction, and an atom S (Fig. 5) and the concomitant formation of a bond between this atom A and an atom E which will play an intermediate role requires less activation energy than when this cleavage is accompanied by the direct formation of a bond between A and an atom P when this bond must be obtained definitively. The reason for this lower value is that the attraction between atom A and atom E is more powerful than that between A and P, for example, because E

(or an atom in its vicinity) carries a suitable charge which P does not have. In other words, the approach of molecule P with a given energy level initially will not be able to overcome the attraction between atom A and atom S of the molecule to which it belongs unless the energy of vibration of the two atoms A and S represented by the Morse curve in Fig. 2 has reached level w_1. On the other hand, the approach of atom E with the same energy of translation may be able to produce this cleavage when atoms A and S are only at level w_2, since the formation of the more powerful bond between A and E will make it possible to furnish the corresponding work $\Delta w = w_1 - w_2$. Due to the stronger attraction between A and E, the basic energy level (at the experimental temperature) of bond A-E is lower, by a quantity exactly equal to Δw, than if this work had not been accomplished. In a subsequent step, P should replace E to yield the final product. Then in order to bring all the atoms involved into the transition state in which atom A is in equilibrium with atom E and atom P, weakening of the bond between A and E will require work, one part of which will compensate for the work saved, Δw, and will be at least equal to this work according to the best hypothesis. There will have been no economy of activation energy; the opposite may actually be true, since the substitution of P for E will again require a level of activation energy which will generally be greater than Δw. However, the total energy will have been supplied in at least two steps.

There thus appears to be no means of diminishing the activation energy required to overcome electrostatic interactions.
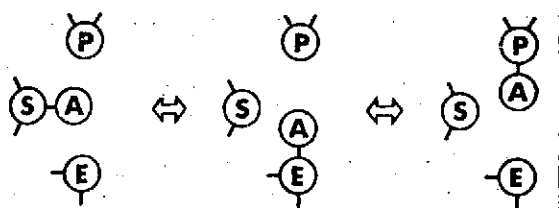

Fig. 5. Formation of a temporary bond between an atom belonging to the substrate and an intermediate molecule (an enzyme) which may facilitate its passage to a receptor molecule P (see text).

Mechanical Work Which May Be Avoided

Such is not the case in attempting to remove another type of
obstacle: the steric hindrance of the molecules. It may happen
that atoms not involved in the reaction prevent it from occurring
by being located between atoms which are to be bonded (atom G
in Fig. 4) or by maintaining contact between atoms which should
separate. Thus these atoms must be removed from the positions
they initially occupy. The work necessary for this displacement
may depend on the "path" which will be followed to the extent that
this work is purely mechanical. In this type of case it fre-
quently occurs that two extremely dissimilar processes leading to
the same result have very different demands for free energy. A
simple example is that of certain types of puzzles consisting of
metal rings and rods intertwined in such a way that it does not
appear possible to remove one element, a ring, for example,
without breaking or at least deforming the puzzle, while in
reality the ring may be removed without applying any force if a
series of maneuvers are performed in which certain rods are
passed through one or several rings in a predetermined order.
In other words, the process of separation would consist in fur-
nishing the high amount of energy necessary to deform the ring,
while it is possible to obtain the same result with a much lower
energy input, "using another path," with the expenditure of
organization work which, it is true, presupposes a decrease in the
entropy of the system. However, this entropy variation may be
slight if the system is well designed. Much more common examples
from day-to-day experience might be cited: less energy is required
to turn a key in a lock than to break down a door.

It is possible to avoid expending any work in cases of this   /29
sort, since the process does not involve bringing the molecules
to a higher energy level, but only eliminating mechanical resis-
tance and not overcoming it. According to the laws of

thermodynamics, in the first eventuality energy must necessarily be supplied to the system, while in the second case it will theoretically be possible to proceed without any expenditure of free energy under conditions of reversibility.

It may easily be conceived that one of the modes of intervention of an enzyme consists in eliminating steric obstacles by bringing about appropriate relative displacements of the obstructing atoms analogous to those made in solving the ring and bar puzzle. Its action in turning away the obstructing atoms consists in guiding the movement of these atoms along a "path," requiring virtually no work. The possibility that a reaction may be facilitated by this type of intervention is undeniable, given that the obstacles presented by certain atoms are purely mechanical. This is due to the fact that an obstacle has a preferred direction in which it is able to move with a low expenditure of energy only when it is not kept in place by electrostatic interaction. It is not possible to avoid the work necessary to overcome gravitational interactions, but interactions of this type are negligible in the problems being considered here.

In practice, atoms between which the bonds to be broken in a given molecule are located, or atoms to which new groups of atoms must be bonded, are always located at the surface of the molecule or in a pocket accessible to the groups of atoms which are to react. Even when access to this pocket is not completely free, it seems unlikely that the removal of atoms which hinder the reaction only by their physical presence could require more than a few hundred calories of energy, that is, a quantity of energy which is negligible in comparison to that necessary to overcome electrostatic interactions.

Effect of Fractioning the Demand for Activation Energy

The intervention of a puzzle type mechanism is not adequate to explain how an enzyme can weaken bonds between atoms or encourage the interpenetration of electron clouds.

It is impossible to diminish the sum total of energy to be supplied, except in regard to the small fraction of energy used to remove mechanical obstacles. It thus appears that in any case, it is necessary to supply the molecules which are to react with a minimum of appreciably the same quantity of energy as that which would be necessary if the reaction were occurring in the absence of a catalyst.

For a single level of thermal energy fluctuations, the presence of an additional type of molecule could well furnish additional energy to the reacting molecules, but the establishment of additional interactions with these molecules will generally require activation energy. Thus it is not possible generally to exclude the idea that the action of an enzyme will decrease the energy of "activation" which must be supplied to the whole from one moment to the next. What next must be considered are the conditions under which this energy may be furnished.

We have observed that, even in the absence of an enzyme, the hydrolysis reaction is a complex reaction; it is necessarily all the more so since, as is the case when it is catalyzed by an enzyme, the hydrogen atom is surrendered to the nitrogen atom by a molecule which serves as an intermediary. Some parts of the reaction may occur simultaneously, while others may or must be preceded by a preparatory step; in addition, all the molecules or groups of atoms involved must have a favorable configuration.

The basic role of an enzyme must be to assure satisfactory coordination of all the partial operations. This coordination

must partly consist in effecting a satisfactory spatial arrangement of the molecules, but, more importantly, it should permit fragmentation of the activation energy supplied.

It is obvious that the fractioning of a reaction among defined substances considerably accelerates its rate due to the exponential decrease in the number of molecules as a function of their energy level. However, when one modifies the initial conditions by introducing into the medium an enzyme which participates in the reaction, the concentration of this enzyme becomes a factor in the expression for the rate of the reaction. As a result, due to the enzyme concentrations, which, under experimental conditions in which the rate may conveniently be measured, are on the order of 10^{-7} M or even less, the rate of the reaction will actually be increased only if the slowest step requires an   amount of activation energy which is at least approximately 10 kcal less than that required for the uncatalyzed reaction (cf. table above).

It should be noted, however, that one may not simply assume that the overall rate of the reaction is limited by the steps requiring the highest free energy of activation, since the rate of each step is simultaneously a function of the corresponding activation energy and the concentration of the reacting substances. Now, for a given concentration of initial substances, the concentration in any intermediate stage depends on the rate of the previous steps leading to the present state. The apparent overall rate of the sum total of reactions, measured on the basis of the rate of disappearance of one of the initial products or the rate of appearance of the final products, thus necessarily depends in complex fashion on the activation energies required by all the steps.

It is impossible to set up a general rule, but it is interesting to consider examples for activation energy values
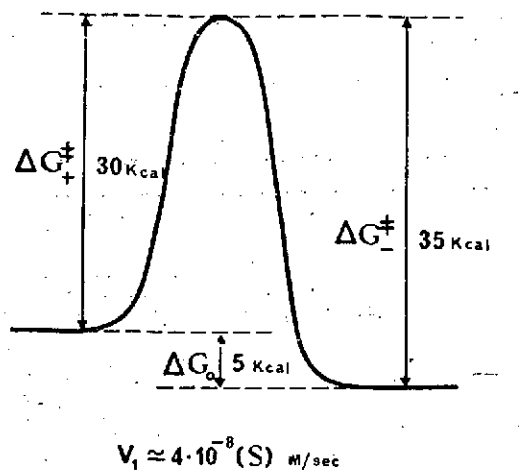
Fig. 6.   Crossing of a single activation enthalpy barrier.

In the figure: $\Delta G_+^{\ddagger}$ 30 Kcal, $\Delta G_-^{\ddagger}$ 35 Kcal, ΔG_0 5 Kcal, $V_1 \simeq 4 \cdot 10^{-8} (S)$ m/sec

whose order of magnitude is that required for steps which could be revealed in reactions catalyzed by enzymes at normal concentrations. A comparison will thus be made of the rates of a single hydrolysis reaction, depending on whether it occurs unaided in one step, or in the presence of an enzyme in two or three steps, using an identical level of total activation energy, since we have seen that one cannot expect to decrease the minimum work required to overcome the electrostatic interactions which in a general view constitute the largest part of the reaction. It will be assumed that the hydrolysis is exergonic and corresponds to a negative variation in standard free enthalpy, ΔG_0. It will also be assumed that it has been possible to obtain conditions such that the concentrations (S) of the initial form and (P) of the final form remain constant. The reaction occurs under stationary conditions. Its rate is constant.

We will first calculate the rate of the reaction in the absence of a catalyst, assuming that in this case it will occur in a single step:

$$S \underset{k_-}{\overset{k_+}{\rightleftarrows}} P$$
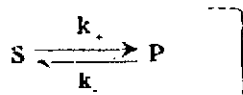
The rate of the reaction as a function of the concentration is

$$V_1 = -\frac{d(S)}{dt} = \frac{d(P)}{dt} = k_+ (S) - k_- (P),$$

or, as a function of the free enthalpies of activation,

$$V_i = \frac{kT}{h}\left[e^{-\Delta G_+^{\ddagger}/RT} (S) - e^{-\Delta G_-^{\ddagger}/RT} (P) \right]$$

A comparison of this theoretical equation with the equations which will be obtained subsequently would furnish no usable information, since the ratio of the rates in one or several steps depends heavily on the numerical values of the activation energies. Arbitrary values will thus be used, assuming that we have here $\Delta G_+^{\ddagger} = 30$ kcal and $\Delta G_-^{\ddagger} = 35$ kcal, and thus $\Delta G_0 = -5$ kcal (Fig. 6) according to the table given above; thus:

$$v_i = \frac{kT}{h} [7.10^{-23} (S) - 1{,}4.10^{-25} (P)].$$

If the concentration of P is on the same order as that of S, the second term in brackets is negligible in comparison to the first on an initial approximation. At 20°C the rate of the hydrolysis is thus

$$v_1 \simeq 5{,}7.10^{12} \times 7.10^{-23} (S) = 4.10^{-8} (S).$$

With substrate concentrations of less than 10^{-3} M, commonly used in vitro, the rate of such a reaction would be virtually zero.

When the reaction is catalyzed by an enzyme it occurs in at least two steps. Assuming the conditions to be those of the simplest case, the diagram for the reaction is

$$S + E \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} P + E$$
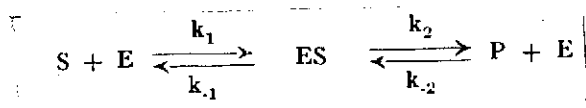
Since we have assumed the system to be stationary, the ES complex concentration is constant, making it possible to write the rate of the reaction as:

$$V_{E2} = \frac{k_1 k_2 (E)(S) - k_{-1} k_{-2} (E)(P)}{k_{-1} + k_2}.$$

where (E) is the free enzyme concentration.

If the sum of activation energies is the same as in the un-catalyzed, single-step reaction -- $\Delta G_1^{\ddagger} + \Delta G_2^{\ddagger} = \Delta G_{+}^{\ddagger}$ -- the rate may be written in the form

$$V_{E2} = \frac{V_1(E)}{e^{-\Delta G_{-1}^{\ddagger}/RT} + e^{-\Delta G_{2}^{\ddagger}/RT}}.$$

It should be noted that the numerator is equal to the rate of the single-step reaction multiplied by (E), and that the denominator includes only the activation energies corresponding to the disappearance of the intermediate complex.

The denominator decreases as $\Delta G_1^{\ddagger}$ and $\Delta G_2^{\ddagger}$ increase, that is, as the intermediate complex remains stable. In practice, the denominator is always much less than 1, since at ordinary temperatures $e^{-\Delta G^{\ddagger}/RT}$ becomes less than 0.5 once $\Delta G^{\ddagger}$ is slightly higher than 400 cal.

The free enzyme concentration (E) is linked to the total enzyme concentration $(E)_t$ by the equation

$$\frac{(E)_t}{(E)} = 1 + \frac{e^{-\frac{\Delta G_1^{\ddagger}}{RT}}(S) + e^{-\frac{\Delta G_2^{\ddagger}}{RT}}(P)}{e^{-\frac{\Delta G_1^{\ddagger}}{RT}} + e^{-\frac{\Delta G_2^{\ddagger}}{RT}}}.$$

It should be noted that this ratio is independent of the total enzyme concentration.

It is not possible to set up a general rule making it possible to predict the ratio of the rate of the reaction as it occurs in two steps to its rate if it were to occur in one step, but it is possible to conceive of a few specific cases, which will give some idea of the possible results of a change from a one-step reaction to a two-step reaction.

Let us first consider the ideal simple case in which the reaction breaks down into two identical steps requiring the same free activation energy.

$$\text{If} \quad \Delta G_1^{\ddagger} = \Delta G_2^{\ddagger} = 15 \text{ Kcal}$$
$$\text{and} \quad \Delta G_{-1}^{\ddagger} = \Delta G_{-2}^{\ddagger} = 17{,}5 \text{ Kcal} \quad \text{(fig. 7),}$$
$$v_{E2} \simeq v_1 \, (E) \times 1{,}2 \times 10^{11}.$$

It may easily be calculated that for a substrate concentration on the order of 10^{-3} M, the free enzyme concentration will differ by only one thousandth of the total concentration.

With an enzyme concentration $(E)_t$ on the order of 10^{-7} M, one would have

$$v_{E2} \simeq v_1 \times 10^4,$$

The assumption of two steps requiring appreciably the same activation energy is not very likely in reality in the case of an enzymatic reaction in which the initial step is the fixation of the enzyme on the substrate. If we therefore assume that $\Delta G_1^{\ddagger}$ is much smaller than $\Delta G_2^{\ddagger}$, assuming, for example, that $\Delta G_1^{\ddagger} = 4$ kcal and $\Delta G_2^{\ddagger} = 26$ kcal, with $\Delta G_{-1}^{\ddagger} = 7$ kcal and $\Delta G_{-2}^{\ddagger} = 28$ kcal (Fig. 8), the result is

$$v'_{E2} \simeq v_1 \, (E) \, 1{,}5.10^5.$$

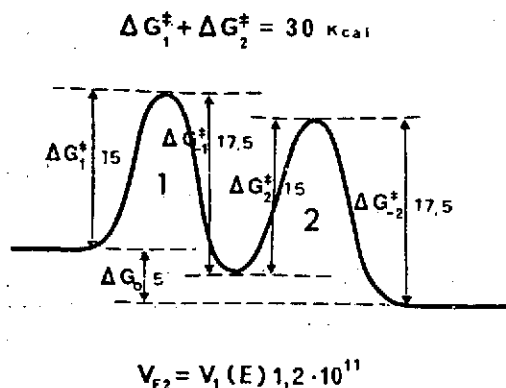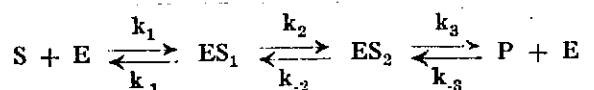$$\Delta G_1^{\ddagger} + \Delta G_2^{\ddagger} = 30 \text{ Kcal}$$

Fig. 7. Crossing of two successive activation enthalpy barriers of the same height, whose sum is equal to the height of the barrier given in Fig. 6.

in other words, the rate of the reaction will be slowed if there is not an extremely heavy enzyme concentration. This is as we predicted for such a small difference between the activation energy of the slowest step and the activation energy of the reaction occurring in a single step.

Although the order of magnitude of the rate of the reaction basically depends on the step requiring the highest amount of activation energy, this step may not actually be considered the sole limiting factor. It may easily be calculated that with the last values adopted, the existence of an initial step reduces the rate to 1/30th of that which would be calculated on the basis of only a single step requiring 26 kcal.

An enzymatic reaction definitely always occurs in more than two steps. Let us consider the case in which our hydrolytic reaction would occur in three steps, following the diagram

$$S + E \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} ES_1 \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} ES_2 \underset{k_{-3}}{\overset{k_3}{\rightleftarrows}} P + E$$

The expression for the rate of the reaction under stationary conditions may easily be calculated. If the sum of the activation energies for each of the steps is equal to the activation energy of the reaction occurring in a single step, $\Delta G_1^{\ddagger} + \Delta G_2^{\ddagger} + \Delta G_3^{\ddagger} = \Delta G^{\ddagger}$, this expression is written

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$$V_{E3} = \cfrac{V_1\,(E)}{e^{-\frac{\Delta G_{-1}^{\ddagger} + \Delta G_3^{\ddagger}}{RT}} + e^{-\frac{\Delta G_1^{\ddagger} + \Delta G_3^{\ddagger}}{RT}} + e^{-\frac{\Delta G_2^{\ddagger} + \Delta G_3^{\ddagger}}{RT}}}$$

As in the case of the reaction occurring in two steps, the numerator of the expression for the rate is equal to the rate of the reaction occurring in a single step multiplied by the free enzyme concentration. The denominator contains the activation energies for the reaction involving the disappearance of the intermediate products.

The free enzyme concentration (E) is linked to the total enzyme concentration $(E)_t$ by the equation

$$\frac{(E)_t}{E} = 1 + \cfrac{(S)\left\{ e^{-\frac{\Delta G_1^{\ddagger} + \Delta G_2^{\ddagger}}{RT}} + e^{-\frac{\Delta G_1^{\ddagger} + \Delta G_3^{\ddagger}}{RT}} + e^{-\frac{\Delta G_1^{\ddagger} + \Delta G_3^{\ddagger}}{RT}} \right\} + (P)\left\{ e^{-\frac{\Delta G_3^{\ddagger} + \Delta G_2^{\ddagger}}{RT}} + e^{-\frac{\Delta G_3^{\ddagger} + \Delta G_2^{\ddagger}}{RT}} + e^{-\frac{\Delta G_3^{\ddagger} + \Delta G_1^{\ddagger}}{RT}} \right\}}{e^{-\frac{\Delta G_{-1}^{\ddagger} + \Delta G_2^{\ddagger}}{RT}} + e^{-\frac{\Delta G_1^{\ddagger} + \Delta G_3^{\ddagger}}{RT}} + e^{-\frac{\Delta G_2^{\ddagger} + \Delta G_3^{\ddagger}}{RT}}}$$

It would be possible to accelerate or slow the rate of the reaction depending on the values of the different activation energies.

It would be possible to compute the rate of the reaction for three identical steps, but our knowledge of the steps of an enzymatic reaction seems rather to indicate that one must first have an initial step requiring a moderate amount of activation energy, followed by at least two principal steps requiring a higher amount of energy [6, 7]. The steps are probably still more numerous, but very little is known about them. We will limit ourselves to three, arbitrarily assuming $\Delta G_1^{\ddagger} = 4$ kcal and $\Delta G_2^{\ddagger} = \Delta G_3^{\ddagger} = 13$ kcal, with $\Delta G_{-1}^{\ddagger} = 7$ kcal and $\Delta G_{-2}^{\ddagger} = \Delta G_{-3}^{\ddagger} = 14$ kcal (Fig. 9). In this case the denominator is appreciably

equal to approximately $2 \cdot 10^{-15}$. The rate of the reaction occurring in three steps is equal to the rate of the reaction occurring in a single step multiplied by $2 \cdot 10^{14}$ (E).

Given the values of $\Delta G^{\ddagger}$, the ratio of the total enzyme concentration to the free enzyme concentration is in practice reduced to

$$\frac{(E)_t}{(E)} \simeq 1 + 3.10^2 \, (S)$$

For a substrate concentration on the order of 10^{-3}, it is still possible to confuse the free enzyme concentration with the total concentration on a first analysis. With an enzyme concentration on the order of 10^{-7} M, the rate of the enzymatic reaction would be 10 million times faster than that of the one-step reaction without catalyst.

Additional steps could be added, and one would find that it is possible to obtain still further acceleration of the overall reaction on the condition that the activation energy required by each step be at least 10 kcal lower than the activation energy for the reaction in the absence of an enzyme.

The possibility of parallel steps should also be considered: for example, the quasi-simultaneous weakening of two bonds belonging to two groups of atoms which in practice are still independent of each other; only the slower process will be the rate-determining step.

Conclusion

A reaction catalyzed by an enzyme is always a complex process, as has been noted from the beginning. In an attempt to analyze this process, partial reactions may be distinguished

arbitrarily. These are successive steps, each of which is characterized by a marking phenomenon, the fixation of molecules on each other, the weakening and cleavage of a bond, or the interpenetration of electron clouds followed by the establishment of a bond. In reality, any reconstruction of the distribution of electrons in the proximity of an atom affects the distribution around all the neighboring atoms.

It is almost certain that the more or less stable inter-
mediate steps which may be detected in reactions catalyzed by an enzyme are not the same as those through which the molecules pass very quickly in the absence of an enzyme: mere engagement of the enzyme molecule in the reaction necessarily changes the manner in which it occurs. Thus the intervention of an enzyme definitely results in a change of the "path" followed by the reaction. However, using this new path, attractions or repulsions which would exist in any case must be definitively overcome, and the sum of the activation energies which must necessarily be furnished to the total reaction may be much less than that necessary for the uncatalyzed reaction. Tallying the energy required for the process followed due to the intervention of an enzyme, one finds that at least the same amount of "activation" energy must be supplied to the total reaction as in the reaction occurring in the absence of a catalyst and in a single step, since in the final analysis, the same electrostatic interactions must be overcome, making use of the formation of the same bonds. In fact, the same result will be obtained by passing through intermediate states which require the cleavage or formation of additional bonds. The sum of the activation energy to be supplied to the total reaction may perhaps be greater, but part of this energy may be furnished by a coupled intermediate reaction, and especially, the bonds will be reconstituted one after another. Now, appropriate fractioning of the energy input considerably increases the rate of the reaction as a whole due to the exponential decrease in the

number of molecules as a function of their level of thermal ;... agitation.

$$\Delta G_1^\ddagger + \Delta G_2^\ddagger = 30 \text{ Kcal}$$
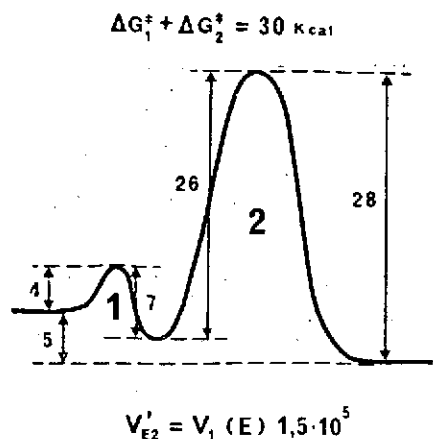

$$V_{E2}' = V_1 \, (E) \, 1{,}5 \cdot 10^5$$

Fig. 8. Crossing of two successive activation enthalpy barriers of different heights whose sum is equal to the heights of the barrier given in Fig. 6.
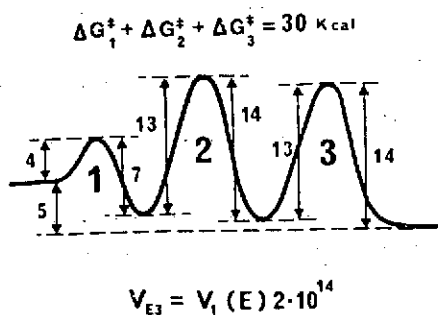
$$\Delta G_1^\ddagger + \Delta G_2^\ddagger + \Delta G_3^\ddagger = 30 \text{ Kcal}$$

$$V_{E3} = V_1 \, (E) \, 2 \cdot 10^{14}$$

Fig. 9. Crossing of two successive activation enthalpy barriers of unequal height whose sum is equal to the height of the barrier given in Fig. 6.

tion of the process involved.

It thus appears that the basic function of the enzyme must be to assure the breakdown of the reaction into a succession of steps, possibly in the first stage due to the fixation of the substrates on the enzyme, one after another, and probably especially due to a gradual transformation of the enzyme-substrate complex successively involving various atoms or groups of atoms, any reconstruction of a local electron distribution having an influence on neighboring atoms. Consistent support for this manner of interpreting the mode of action of enzymes may be obtained from an analysis of X-ray diffraction to determine how the pseudo-substrates come to be attached to the crystallized enzyme and the resultant movements of the lateral chains of the enzyme, in the case of lysozyme [10] and carboxypeptidase [11], for example.

It has thus been found that the statement that an enzyme lowers the energy barrier of the reaction is a totally inaccurate representation. The only definite finding is that

an increase in the rate of the reaction may be observed which is characterized by the rate of disappearance of a substance which was initially present or the appearance of one of the final products. Thus if one finds that the overall rate of the reaction is proportional to the concentration of reacting substances, that is, if it is in the form $v = -k(A)(B)$, the coefficient of proportionality k appears as a rate constant, but this constant is complex: it is an implicit function of the rate constants of several partial reactions playing different roles in the reaction as a whole. A succession of reactions cannot be assimilated by a single reaction occurring according to the elementary process to /34 which the theory of absolute rates (and even the Arrhenius theory) may be applied. In all cases in which several phases may be distinguished in a reaction, the free enthalpy of activation which is calculated on the basis of the complex experimental rate constants (assuming that the rate of the reverse reaction is negligible) is only an <u>apparent</u> energy of activation. It does not correspond to any precise phenomenon, and it is impossible to interpret in physical terms the difference between this enthalpy of activation and that of a single-step reaction which would yield the same final product from the same initial reactants. Specifically, to state that the enzyme lowers the activation energy barrier is thus only an obscure and incorrect way of saying that the rate of the reaction is increased. Although it offers an image, this expression does not translate any phenomenon and provides no initial basis of explanation. It is impossible to speak of the activation energy necessary to cross <u>the</u> energy barrier, since one knows that catalysis is a complex phenomenon and that the catalyzed reaction includes at least two steps (formation of the molecule-catalyst complex and transformation of the molecule), and usually a very large number of steps. In the enzymatic reactions which have been studied, it was possible to reveal the existence of several intermediate compounds.

The basic role of the enzyme is to coordinate the sequence of events in such a way that the activation energy may be supplied little by little, in "small" quantities. This coordinating function depends on the ability of the enzyme to fix the substrates in relative positions which will be favorable for complementarity. This complementarity depends on a geometrical arrangement of the electron clouds of certain specific atoms. Mere fixation of a substrate on an enzyme, which would occur by van der Waals attraction, changes the arrangement of the electron in the substrate and the enzyme and makes it possible for a second step to occur. Any distinction between the site of fixation and the active site is artificial, although it may be useful in an analysis of the complete process. In addition, it does not appear very likely that there are distinct and clearly separated steps. The interactions between electrons and atoms develop gradually, and at the very most, there must be quasi-stationary phases during which the changes in configuration are slow.

There is no mystery in the catalytic capability of enzymes, although there is still much work to be done before all the details of their modes of action are known.

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